

Detection of HBV DNA in Serum Using a PCR-Based Assay

Hau Tim Chung

1. Introduction

Detection of minute amounts of hepatitis B virus (HBV) DNA in the serum using polymerase chain reaction (PCR)-based assay involves extracting the viral DNA from the viral particle in the serum, removing inhibitors of PCR, performing the PCR, and detecting the PCR product. PCR is an extremely sensitive assay, and preventing cross contamination is an important part of the assay.

1.1. HBV DNA Extraction from Viral Particles and Removal of Inhibitor of PCR

HBV DNA in viral particles in serum is covered by a coat of hepatitis B core antigen (HBcAg) particles and a lipid coat with hepatitis B surface antigen (HBsAg) in it. Removal of the HBcAg and the HBsAg with the lipid coat can be easily accomplished by treatment with a detergent or alkali. However, there are many inhibitors of the PCR reaction in the serum. Deproteinization removes most of these inhibitors and it forms the basis of the procedure being described and used by the author. Alternatively, PCR can also be performed from DNA extracted directly from serum.

1.1.1. Proteinase K/Phenol/Phenol Chloroform/Ethanol Precipitation

Extraction of HBV DNA from serum is a tedious procedure, and its yield is variable, which directly affects the sensitivity or detection limit of the assay. Moreover, each step in the procedure creates a risk for cross contamination. However, it will also serve as a concentration method. The sensitivity of the assay can be improved by simply increasing the amount of serum used for the extraction. The volume limit of the actual PCR, which is a result of the need to change the temperature at a rapid pace, does not count here. The negative strand of the HBV DNA molecule is covalently bound to a small piece of protein, and thus the whole molecule may stay in the interface if the proteinase K digestion is not performed well. This is one of the many problems that affect the yield

in HBV DNA extraction using proteinase K/phenol/phenol chloroform/ethanol precipitation. In a well-digested specimen, the interface between the aqueous and phenol phase should be almost nonexistent. The presence of any significant amount of interface will drastically reduce the yield and thus affects the detection limit of the assay.

1.1.2. Alkali Denaturization

PCR can also be performed using neat deproteinized serum that has been treated with a denaturing agent to release the nucleic acid from the lipid and protein coat. Proteinase K digestion is one of the methods for removing protein, but this process can also be achieved by alkali treatment of the serum and heat denaturing of the protein. PCR can be performed in the same tube with the denatured protein spun down. This method reduces dramatically the number of steps needed in the procedure and saves time, labor, and cost. More important, fewer steps and tube changes also reduce the risk of cross contamination.

1.2. Performing PCR and Detection of Its Products

PCR can be performed in the standard way using the deproteinized neat serum. When two sequential PCR steps of 30 cycles each are used with two sets of nested primers, the level of DNA can be amplified from as low as one molecule to a level that can easily be detected using ethidium bromide staining of a polyacrylamide gel. This method is much easier and less expensive than using a more sensitive detection method, such as Southern blotting, to detect a smaller amount of product from a single round of 30-cycle PCR. The turnaround time of the protocol described below is within one working day, compared with at least five for PCR-Southern blotting. It also removes the need to work with radioisotopes.

1.2.1. Choice of Primers

All published sequences of the hepatitis B virus (*1–10*) were aligned using a computer program. The HBV sequences have a reasonably conserved sequence among various isolates. There are only a few regions with significant variations: 851–999, 1977–2203, 2513–2815, and 2852–57 (HBV DNA sequence numbering system is according to Galibert et al. [1]). Regions of fewer than 300 base pairs in length of highly conserved regions were deemed suitable to be amplified using PCR and will achieve a high yield. This region has to be framed by two pairs of perfectly conserved short sequences, each about 20 nucleotides long, to be used as pairs of nested primers. One set of nested pairs of primers was chosen from the surface-antigen-coding region and another from the core-coding region. Running two PCR's for each specimen using two different sets of nested primers reduces the theoretical risk of variant viruses failing to be detected if one of the primers does not match the target sequence. It may also pick up cases of false-positive results caused by inadvertent cross contamination by PCR products from previous reactions.

1.2.2. Sequence of the Chosen Primers

Nested primer sets for surface-antigen-coding region:

Primer set for first PCR:

Primer 1: CCTGCTGGTGGCTCGAGTTC (58–77)

Primer 2: CAAACGGGCAACATACCTTG (486–467)

Primer set for the second PCR:

Primer 3: ACATCAGGATTCCTAGGACC (169–188)

Primer 4: CGCAGACACATCCAGCGATA (389–370)

These sets of primers used in a nested PCR will give a product of 221 base pairs in length.

Nested primer sets for the core-antigen-coding region:

Primer set for the first PCR:

Primer 5: GGAGTGGGATTCGCACTCC (2269–2288)

Primer 6: ATACTAACATTGAGATTCCC (2457–2438)

Primer set for the second PCR:

Primer 7: AGACCACCAAATGCCCTAT (2299–2318)

Primer 8: GATCTTCTGCGACGCGCGCA (2429–2410)

These sets of primers used in a nested PCR will give a product of 131 or 137 base pairs in length, depending on the subtype of the HBV target.

1.3. Prevention of Cross Contamination

Cross contamination can be caused by HBV DNA present in the laboratory environment, on bench tops, on utensils, and as aerosol within the piston mechanism of pipetting instruments left from previous experiments performed in the same laboratory. More important, PCR products are short DNA sequences that can survive in the environment for a long period and are potential target sequences that will give a positive result in an assay. The number of copies of these PCR products totals millions- to trillions-fold that of HBV DNA handled in a clinical specimen and thus has a much higher risk of cross contamination. The following steps are used to reduce the chance of cross contamination:

1. Most instruments should be used only once when collecting a blood specimen from the subject. They include needles, needle holders, specimen tubes, and syringes. Gloves should be changed in between subjects, and extra care should be taken to avoid soiling of the tourniquet by blood.
2. Care should be taken to avoid contamination of the laboratory environment or cross contamination when centrifuging blood and separating serum from the specimen. Serum should be sucked out using a single-use Pasteur pipet with bulbs attached. Reusable bulbs cannot be used.
3. Consideration in avoiding cross contamination should be observed in storing specimens for future analyses, when thawing the specimen, and when aliquoting specimens for assay. Serum should not be stored in Eppendorf tubes with flip-open lids. Tiny amounts of serum always get into the lid when it is inverted for mixing after thawing and contaminate the glove

used to open it. Serum should be stored in screw-top tubes designed in such a way that serum will not get onto the glove when it is handled, inverted for mixing, or opened.

4. Procedures before PCR should be physically isolated from those after PCR. Ideally, they should be performed on different benches using different sets of instruments, in particular, pipettors. Gloves should be changed in between handling samples in the steps before and after PCR.
5. All solutions should be prepared using single-use utensils. They are prepared in large lots, aliquoted to portions sufficient for a single run, and stored in a refrigerator or freezer until used. Unused portions are discarded. The only exception to this rule is the *Taq* polymerase enzyme. It is added into the PCR mix just before it is dispensed into the reaction tube.
6. All pipetting should be performed using either a positive displacement pipet (Microman, Gilson, France) or an ordinary pipettor with filtered pipet tips (United States Biochemical Corps., Cleveland, OH, USA). This approach was found to be the single most important step in preventing cross contamination, with the vast majority of cases containing aerosol contaminations.
7. All PCR products should be disposed of carefully to avoid contaminating the laboratory environment. The protocol described in the following paragraphs used a minimum number of steps, a minimum number of pipettings, and a minimum number of tubes. Pipet tips, Eppendorf tubes, electrophoresis apparatus, the polyacrylamide gel, and the ultraviolet (UV) light box used to view the gel are potential sources of PCR products that could cause cross contamination. Eppendorf tubes are disposed of with lids closed, and pipet tips and gel are disposed of carefully, making sure the bench top and environment are not contaminated. Electrophoresis solutions are discarded carefully into the sink and flushed with ample amounts of water. The electrophoresis apparatus is washed with plenty of water. The UV light box can be wiped with 1 *N* HCl and neutralized with 1 *M* Tris-HCl pH 7 5 minutes later. Gloves are changed after handling these steps.

2. Materials

1. 1 *N* NaOH.
2. Tris-HCl/HCl: mixture of equal volume of 2 *M* Tris HCl, pH 8.3 and 2 *N* HCl.
3. PCR mix 1–2: 12.5 *mM* Tris-HCl, pH 8.3, 62.5 *mM* KCl, 1.875 *mM* MgCl₂, 250 μ *M* each of the four deoxyribonucleotides (dATP, dTTP, dCTP, and dGTP), 1.25 μ *M* each of primer 1 and primer 2.
4. PCR mix 3–4: same as PCR mix 1–2, but use primer 3 and primer 4 instead of primer 1 and primer 2.
5. PCR mix 5–6: same as PCR mix 1–2, but use primer 5 and primer 6 instead of primer 1 and primer 2.
6. PCR mix 7–8: same as PCR mix 1–2, but use primer 7 and primer 8 instead of primer 1 and primer 2.
7. *Taq* polymerase enzyme.
8. 6X loading buffer: 15% Ficoll 400/0.15% bromphenol blue.

3. Methods

The following protocol utilizing alkali denaturization was used regularly by the author and will work, except if the specimen is heavily hemolyzed before separation (11–14).

1. Serum has to be separated from the blood specimen in a timely fashion to avoid hemolysis.
2. Put 10 μ L of serum into a 500- μ L Eppendorf tube.

3. Add 1 μL of 1 *N* NaOH solution.
4. Cover with 10 μL of mineral oil.
5. Heat to 37°C for 1 hour.
6. Add 1 μL of Tris-HCl/HCl. Care has to be taken that the solution is added into the aqueous phase of the tube and is not floating on the top of the mineral oil layer as a result of surface tension.
7. Heat to 98°C for 5 min, Protein will be denatured and come out of the solution as a yellowish precipitate.
8. Centrifuge in a microcentrifuge for 5 min. The denatured protein precipitate will stay in the bottom of the tube and will not interfere with the subsequent reaction.
9. Add *Taq* polymerase enzyme into a volume of PCR mix 1–2 just enough for the total number of tubes in the run. The final amount of enzyme should be 2.5 U per 40 μL of PCR mix.
10. Add 40 μL of solution from step 9 into the aqueous phase of specimen in step 8. There is no need for mixing, and care has to be taken not to disturb the protein precipitate at the bottom of the tube.
11. Put the Eppendorf tube into a PCR machine.
12. Run 30 cycles of PCR, each consisting of 54 seconds at 94°C, 1 minute at 50°C, and 1 minute at 72°C.
13. When PCR in step 12 is about to finish, add *Taq* polymerase enzyme into a volume of PCR mix 3–4 just enough for the total number of tubes in the run. The final amount of enzyme should be 2.5 U per 40 μL of PCR mix.
14. Set up the same number of Eppendorf tubes as the number of specimens run in step 2. Fill each of them with 40 μL of solution from step 13 and cover with 10 μL of mineral oil.
15. Pipet 10 μL of the PCR product from step 12 into each of the tubes from step 14.
16. Run 30 cycles of PCR, each consisting of 54 seconds at 94°C, 1 minute at 50°C, and 1 minute at 72°C.
17. Add 10 μL 6X loading buffer into each tube. Mix by pipetting and load 10 μL into a 5% polyacrylamide gel using the same pipet tip. Run electrophoresis and stain with ethidium bromide. Lanes with staining at 221 base pairs are positive.
18. Each run should include negative and positive controls. The positive control is made by diluting a positive serum with a known amount of hepatitis B virus (determined using dot blot hybridization) using a negative serum. The concentration of the positive control should be about 1–2 molecules of HBV DNA (the author used the equivalent of about 5×10^{18} g HBV DNA) per 10 μL .
19. The above steps are also run using the core protein-coding region primers by substituting PCR mix 1–2 in step 9 with PCR mix 5–6 and PCR mix 3–4 in step 13 with PCR mix 7–8. In step 17, lanes with staining at 131 or 137 base pairs are positive.
20. One way of controlling for the absence of PCR inhibitors in each specimen is to run a positive control for each specimen by spiking it with a known positive serum.

References

1. Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., and Charnay, P. (1979) Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* **281**, 646–650.
2. Pasek, M., Goto, T., Gilbert, W., et. al. (1979) Hepatitis B virus genes and their expression in *E. coli*. *Nature* **282**, 575–579.
3. Valenzuela, P., Gray, P., Quiroga, M., Zaldivar, J., Goodman, H. M., and Rutter, W. J. (1979) Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen. *Nature* **280**, 815–819.

4. Valenzuela, P., Quiroga, M., Zalvidar, J., Gray, P., Rutter, W.J. (1980) The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. In: Fields, B.N., Jaenisch, R. (eds.) *Animal Virus Genetics*, 57–70.
5. Ono, Y., Onda, H., Sasada, R., Igarashi, K., Sugino, Y., and Nishioka, K. (1983) The complete nucleotide sequences of the cloned hepatitis B virus DNA: subtype adr and adw. *Nucleic Acids Res.* **11**, 1747–1757.
6. Fujiyama, A., Miyanohara, A., Nozaki, C., Yoneyama, T., Ohtomo, N., and Matsubara, K. (1983) Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. *Nucleic Acids Res.* **11**, 4601–4610.
7. Pumpen, P.P., Kozlovskaya, T.M., Borisova, G.L., et al. (1984) Synthesis of the surface antigen of hepatitis B virus in *Escherichia coli*. *Dokl. Biochem. Sect.* **271**, 246–249.
8. Kobayashi, M., and Koike, K. (1984) Complete nucleotide sequence of hepatitis B virus DNA of subtype adr and its conserved gene organization. *Gene* **30**, 227–232.
9. Bichko, V., Dreilina, D., Pushko, P., Pumpen, P., and Gren, E. (1985) Subtype ayw variant of hepatitis B virus. *FEBS Lett.* **185**, 208–212.
10. Lo, S.J., Chen, M.-L., Chien, M.-L., and Lee, Y.-H.W. (1986) Characteristics of pre-S2 region of hepatitis B virus. *Biochem. Biophys. Res. Commun.* **135**, 382–388.
11. Chung, H.T., Lai, C.L., and Lok, A.S.F. (1989) Hepatitis B virus has an etiological role in the pathogenesis of cirrhosis in patients positive for anti-HBs or anti-HBc. *Hepatology* **10**, 577.
12. Chung, H.T., Lok, A.S.F., and Lai, C.L. (1993) Re-evaluation of alpha-interferon treatment of chronic hepatitis B using polymerase chain reaction. *J. Hepatol.* **17**, 208–214.
13. Chung, H.T., Lee, J.S.K., and Lok, A.S.F. (1993) Prevention of post-transfusion hepatitis B and C by screening for antibody to hepatitis C virus and antibody to hepatitis B core antigen. *Hepatology* **18**, 1045–1049.
14. Chung, H.T., Lai, C.L., and Lok, A.S.F. (1995) Pathogenic role of hepatitis B virus in hepatitis B surface antigen negative cirrhosis. *Hepatology* **22**, 25–29.

Hepatitis B and D Protocols

Volume 1: Detection, Genotypes, and Characterization

Hamatake, R.K.; Lau, J.Y.N. (Eds.)

2004, 376 p. 110 illus., 1 illus. in color., Hardcover

ISBN: 978-1-58829-105-9

A product of Humana Press